Physiological and Pathological Characteristics of Virulent and Avirulent Strains of the Bacterium that Causes Pierce's Disease of Grapevine

D. L. Hopkins

Professor of plant pathology, University of Florida, Agricultural Research and Education Center, Leesburg 32749-0388.

ABSTRACT

Hopkins, D. L. 1985. Physiological and pathological characteristics of virulent and avirulent strains of the bacterium that causes Pierce's disease of grapevine. Phytopathology 75:713-717.

Virulence of strains of Pierce's disease bacteria to grapevine (*Vitis vinifera* 'Carignane') was determined by the multiplication of bacteria in the xylem vessels of inoculated petioles or stem internodes and by their capability to move systemically from vessel to vessel through the plant. Virulent strains reached populations of 10^6–10^7 colony-forming units per centimeter of inoculated petiole and avirulent strains reached only 10^4–10^5 colony-forming units per centimeter. Virulent strains moved systemically from the inoculated internode and infested xylem vessels in all but the youngest grapevine tissue within 3 mo. Avirulent strains did not move beyond the inoculated internode. Weakly virulent strains multiplied more slowly in petioles than virulent ones but eventually reached 10^6 colony-forming units per centimeter, and systemic movement of weakly virulent strains was slower. None of the strains that cause Pierce's disease produced pectolytic, cellulolytic, or proteolytic enzymes in culture. Peroxidase and lysophospholipase were produced; virulent strains produced the most lysophospholipase.

Additional key words: rickettsialike bacteria, xylem-limited bacteria.

Pierce's disease (PD) of grapevine is the major factor in the failure of bunch grapes (*Vitis vinifera* L. and *V. labrusca* L.) in the southeastern United States (9). PD is caused by a small, Gram-negative, xylem-limited bacterium previously referred to as a rickettsialike bacterium (7,12). The PD bacterium was first grown in axenic culture and Koch's postulates were fulfilled in 1978 (3).

The symptoms of PD suggest a dysfunction of the water conducting system (9). Bacterial aggregrates, gums, and tyloses block vessels and probably restrict water movement in grapevines affected by PD (15), but rarely do more than 40% of the xylem vessels in a cross section contain occlusions. Plants show no water stress with this percentage of nonfunctioning vessels (5). However, serial sectioning reveals that there may be enough occlusions over the length of a petiole or leaf vein to produce sufficient water stress to result in the leaf marginal necrosis (MN) symptom, especially if lateral water movement between vessels were also inhibited (11). The PD bacterium also has been reported to produce a phytotoxin that causes MN symptoms in leaves of grape (14). Therefore, PD symptoms could result from a physical blockage of xylem vessels, from a phytotoxin, or a combination of the two.

The virulence of PD isolates from grapevine and other hosts varies from avirulent to highly virulent (10). There also seems to be a loss of virulence of cultured isolates during weekly serial transfers. This results in virulent, weakly virulent, and avirulent strains that may prove useful in studies on mechanisms of pathogenesis. In some cases, we have virulent and avirulent strains of the same isolate. Virulence of PD strains was not determined solely by their ability to infest grapevine tissue (13). Avirulent strains were able to infest the xylem vessels, multiply, and be reisolated at least 5 wk later.

The objective of this study was to quantitatively compare PD strains having different levels of virulence to grapevine for their ability to infest, multiply, move systemically within the plant, and produce extracellular, degradative enzymes that are commonly produced by other vascular wilt-inciting pathogens.

MATERIALS AND METHODS

Strains of the PD bacterium were isolated from leaf petioles of bunch grapes, muscadine grapes (*V. rotundifolia* Michx.), and wild grapes (*V. munsoniana* and *V. riparia*) with PD symptoms and from root or stem segments of blight-affected rough lemon and sweet orange (*Citrus jambhiri* Lush. and *C. sinensis* [L.] Osbeck, respectively) (Table 1). Isolates were single-colony transferred twice by dilution plating. These strains were lyophilized to maintain virulence. Avirulent strains were obtained in some instances after weekly serial transfers for 1 yr or more. Therefore, I had avirulent strains and lyophilized virulent strains from the same original isolation (Table 1).

Evaluation of virulence of strains of the PD bacterium. For inoculum preparation, the PD strains were grown at 28 C on PD2 medium (4) modified by substituting soluble starch for bovine serum albumin. Bacterial inoculum suspensions containing cells from 4- to 6-day-old cultures were prepared in a succinate-citrate-phosphate buffer composed of disodium succinate, 1.0 g/L; trisodium citrate, 1.0 g/L; K_2HPO_4, 1.5 g/L; KH_2PO_4, 1.0 g/L; pH 7.0. The inocula were adjusted to $A_{600 \text{ nm}} = 0.25$ (10^7–10^8 colony-forming units [cfu] per milliliter) with a Bausch and Lomb Spectronic 2000 spectrophotometer. Dilution plating was used to determine that $A_{600 \text{ nm}}$ 0.25 of the inocula equaled 10^7–10^8 cfu/ml.

Rooted green cuttings of cultivar Carignane grape with a minimum of six nodes at inoculation were used in the virulence tests. The pin-pricking inoculation technique (10) was used. A drop (0.02 ml) of inoculum was placed on the first and third internodes from the base of the cutting and the stem was pierced three to five times through the drop with a dissecting needle. The inoculum was pulled into the plant by the negative pressure of the pierced xylem vessels. Inoculated plants were kept in a greenhouse at 28–33 C in the daytime and 20–25 C at night.

Inoculated plants were observed for symptoms of PD every 2 wk for 4 mo after inoculation. Culturing the PD bacterium from petioles was used to confirm visual diagnoses. After 4 mo, severity ratings of PD symptoms were made according to the following arbitrary scale: 0 = no symptoms; 1 = any symptom of PD, such as MN, on the basal leaf; 2 = MN in one-third or less of the leaves; 3 = one-third to one-half of the leaves with MN; 4 = one-half to three quarters of the leaves with MN and dead growing point; and 5 =

100% of the leaves with symptoms or a dead plant. Strains that caused PD with a severity rating of 3, 4, or 5 were considered virulent, those that produced a rating of 1 or 2 were considered weakly virulent, and those with a rating of 0 were considered avirulent.

Bacterial multiplication in petioles and leaf veins. For PD bacterium multiplication studies, one drop of bacterial suspension (10^7–10^8 cells per milliliter) was used to inoculate each grape petiole. At various times after inoculation, populations of PD bacteria were determined from 2-cm-long petiole samples centered on the inoculation point. Samples were surface sterilized in 1% sodium hypochlorite for 3 min and washed in four changes of sterile water. They were then ground in 10 ml of succinate-citrate-phosphate buffer with a mortar and pestle, filtered through cheesecloth, and centrifuged (15 min at 4,000 g) to concentrate the bacteria. The bacteria were resuspended in 2 ml of sodium-citrate-phosphate buffer and quantified by dilution plating on PD2 medium. Values given are the average of three petioles. Samples of leaf midvein 2 cm in length were processed in the same manner.

Movement of PD bacteria from the inoculation point. Rooted green cuttings of cultivar Carignane grapevine were inoculated at two points in the third internode from the base. One drop of inoculum (10^7–10^8 cfu/ml) was applied at each point and inoculated into the plant by pin-pricking as described above. At various time intervals after inoculation, two inoculated plants were sacrificed and the extent of colonization by the PD bacteria was determined. Plants were cut into internodes and petioles and labeled from the base as -2, -1, 0 (the inoculated internode), 1, 2, 3, 4, etc. Internode and petiole samples were surface sterilized in 1% sodium hypochlorite for 3 min and rinsed four times in sterile water. The samples were aseptically cut into 0.5–1.0-cm sections. The sections were squeezed with forceps and the sap exuding from each section was blotted onto modified PD2 medium (4). Since there were very few contaminants, the PD bacteria could be identified visually by colony size and type after 7 days of growth.

Enzyme assays. PD strains were grown in liquid PD2 medium for the preparation of culture filtrates (CF) for enzyme assays. Stationary broth cultures were incubated for 2 days at 28 C and then placed on a shaker for an additional 3–4 days at 25 C. The strains grow at similar rates in culture (13) and these cultures were approximately 10^9 cfu/ml. Bacterial cells were removed by centrifugation at 10,000 g for 20 min. The supernatants were dialyzed against deionized water for 24–48 hr at 4 C. This dialyzed CF was concentrated 5:1 by lyophilization and stored frozen until used in enzyme assays.

Polygalacturonase (PG) activity was determined by a viscosity reduction method (2). Reaction mixtures contained 2 ml of CF and 8 ml of 1% sodium polypectate buffered at pH 5.0 by 0.1 M citrate buffer. The loss in viscosity of the substrate was estimated in Cannon-Fenske size 200 viscometers in a 25 ± 0.1 C water bath.

Polygalacturonate trans-eliminase (PGTE) was assayed by the thiobarbituric acid (TBA) procedure (1). Pectic enzyme production by the PD strains was also studied by observing pit formation on pectin and sodium polypectate gels (8). Loopfuls of bacteria from 5- to 6-day-old cultures on PD2 medium were spotted on the gels, four to a plate, and incubated at 28 C for 6 days.

Cellulase activity was determined by measurement of a loss in viscosity of the substrate similar to the PG assay except 0.21% carboxymethylcellulose was used as the substrate (2).

Proteolytic enzymes were assayed by using hide powder azure. The reaction mixture contained 5 mg of hide power azure, 0.9 ml of 0.05 M tris-HCl buffer (pH 8.0), and 0.1 ml of enzyme solution. Incubation was for 1 hr at 37 C. The mixture was diluted with 4 ml of water, filtered through Whatman #1 filter paper, and $A_{600\,nm}$ was measured.

Acid carboxypeptidase and aminopeptidase activities were assayed by the methods of Nakadai et al (16,17).

The reaction mixture for the peroxidase assay consisted of 1.5 ml of 0.1 M phosphate buffer, pH 5.0; 0.5 ml of 0.02 M guaiacol; 0.5 ml of enzyme preparation; and 0.15 ml of 0.06 M H_2O_2 (18). The addition of H_2O_2 initiated the reaction. Activity was expressed as the change in absorbance per minute at 485 nm.

Phospholipase and lysophospholipase activities were determined by a cup-plate assay based on the method of Doery et al (6). Agar plates were prepared by mixing molten 4% (w/v) agar with an equal volume of 1% soybean lecithin suspension or 0.5% lysolecithin suspension containing 3.2×10^{-3} M $MgSO_4$ and 0.01% (w/v) thiomersal. The mixture was poured into 100-mm-diameter petri plates to give a 5-mm layer of substrate. Wells (8 mm in diameter) were cut in the agar and these were filled with enzyme solution.

In all enzyme assays, commercial enzyme preparations were positive controls and autoclaved commercial enzymes and CFs were negative controls.

RESULTS

Bacterial multiplication in petioles and leaf veins. Virulent, weakly virulent, and avirulent PD strains were all able to infest and multiply at the inoculation site in a grape petiole (Fig. 1). With all three types of strains, the most rapid multiplication occurred 6–10 days after inoculation. The virulent CB-9 reached a maximum of 10^6–10^7 cells per centimeter of petiole at 10–14 days after inoculation and produced leaf marginal necrosis symptoms at 21–28 days. The weakly virulent strain reached a maximum population of 10^6–10^7 cells per centimeter of petiole after 28–35 days and produced symptoms at 56–70 days. The avirulent strain CB-1 reached a maximum population of 10^4–10^5 cells per centimeter after 14 days and then declined, producing no symptoms. Population curves of other strains were similar, except that avirulent strains sometimes could not be recovered after 21–28 days.

To evaluate bacterial multiplication and movement away from the inoculation point in the petiole, leaf midvein multiplication patterns were determined. The virulent strain apparently moved into the leaf vein very quickly since it reached a maximum population approximately 14 days after inoculation of the petiole (Fig. 2). The weakly virulent PD-4 moved into the leaf vein and rapidly multiplied at 28–35 days after inoculation. The avirulent strains, in this case CB-1, did not move from the petiole to the leaf vein.

From the multiplication curves in Fig. 1, populations in the inoculated petiole at 21 days after inoculation would best separate virulent, weakly virulent, and avirulent PD strains. Populations of

TABLE 1. Comparison of strains of Pierce's disease bacteria for multiplication in grapevine and extracellular lysophospholipase

Strain[a]	Source[b]	Virulence[c]	Multiplication[d] (cfu/cm petiole)	Lysophospholipase assay[e]
PD-1	BG	V	3.8×10^7	3
PD-15	BG	V	7.0×10^6	3
CB-9	C	V	5.8×10^6	2
PD80-2	M	V	2.4×10^6	3
CB80-4	C	WV	2.5×10^5	1
PD-12	WG	WV	9.2×10^5	2
PD79-8	WG	WV	2.0×10^4	2
PD80-8	WG	WV	6.0×10^4	2
PD-la	BG	A	7.0×10^2	1
PD-15a	BG	A	0.0	1
CB-1	C	A	9.0×10^4	0

[a] PD-la and PD-15a are strains that became avirulent by serial transfers of PD-1 and PD15, respectively. CB-1 also became avirulent by serial transfer. Culture filtrates for toxin and lysophospholipase assays were all from cultures containing 10^8–10^9 cfu/ml.
[b] BG = bunch grape, C = citrus blight, M = muscadine grape, and WG = wild grape.
[c] V = virulent, WV = weakly virulent, and A = avirulent.
[d] Multiplication of the various strains in grapevine petioles is expressed as cells per cm of petiole 21 days after inoculation.
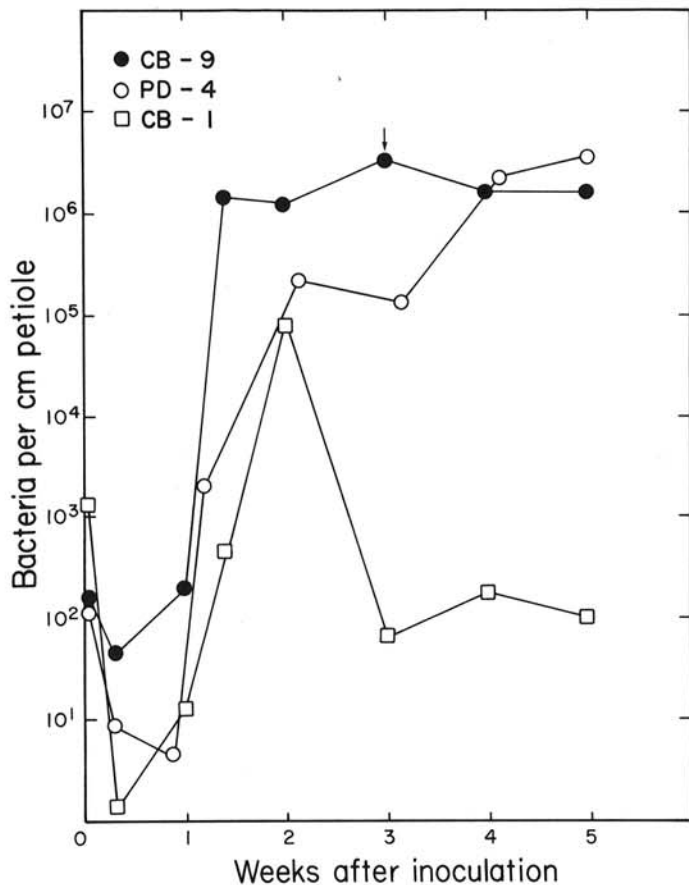[e] Activity was based on a cup-plate assay in which the width and intensity of an opaque band around the sample well was rated on a 0–3 scale, with 0 = no band and 3 = 3- to 4-mm intense band. The substrate gel contained 0.5% lysolecithin.

11 strains in Carignane petioles 21 days after inoculation confirmed this observation (Table 1). Four virulent strains average 1.3×10^7 cells per centimeter, four weakly virulent strains averaged 3.1×10^5 cells per centimeter, and three avirulent strains averaged 3.0×10^4 cells, but this resulted from only one of the three avirulent strains.

Movement of PD bacteria from the inoculation point. Systemic movement of virulent, weakly virulent, and avirulent strains through the grapevine were compared after inoculation of a single internode. Inoculation (0 time) placed bacteria in one to three internodes (Fig. 3). The first detectable movement of bacteria was 3 wk after inoculation when the virulent PD-1 was isolated from five internodes and PD-4 from four internodes. The PD-1 bacteria continued to move up the grapevine for the 8-wk duration of the test, whereas PD-4 was found nine internodes up the vine at 5 wk and remained at that level through 8 wk. The avirulent PD-15 was isolated from the inoculated internode in the 0-time and 1-wk samples, but was not recovered after that.

Movement of bacteria up the grapevine and into the petioles was similar to movement determined by isolation from internodes. The first obvious upward movement of the virulent PD-1 occurred at 3 wk, and the bacteria continued to move until the end of the 8-wk test when PD-1 was found in the leaf petiole at the 15th node above the inoculated internode (Fig. 4). The weakly virulent PD-4 moved into the petiole at the 6th node above the inoculation point at 4 wk after inoculation but moved no further. The avirulent PD-15 was never isolated from a petiole.

Enzyme activity. Concentrated CFs of various virulent, weakly virulent, and avirulent PD strains were assayed for polygalacturonase and polygalacturonate trans-eliminase activity. None of the CFs had any pectolytic activity. None of the PD strains produced pits on pectin or sodium polypectate gels. PD strains were also grown in liquid PD2 medium amended with the following carbon sources: pectin (8 g/L), polygalacturonic acid (8 g/L), cellobiose (1 g/L), arabinose (1 g/L), xylose (1 g/L), mannose (1 g/L), D-galacturonic acid (1 g/L), galactose (1 g/L), or glucose (1 g/L). These cultures grew to 10^8-10^9 cfu/ml, but all of the CFs were also negative for pectic enzymes.
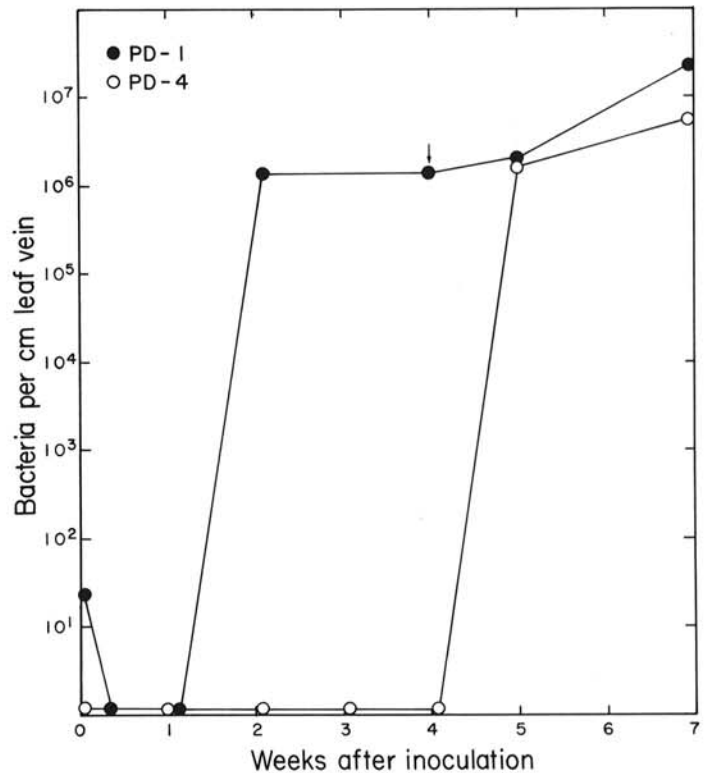
Fig. 2. Growth curves of virulent (PD-1) and weakly virulent (PD-4) strains of the Pierce's disease bacterium in the midvein of leaves inoculated in the petiole at time 0 with a drop of 10^7-10^8 cells per milliliter of bacterial suspension. ↓ = time of first appearance of leaf marginal necrosis symptoms with PD-1 and symptoms were produced at 56 days with PD-4.
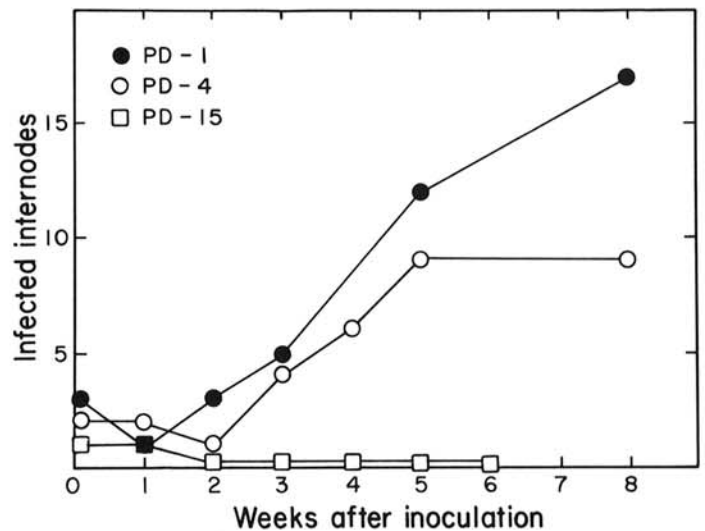

Fig. 1. Growth curves of virulent (CB-9), weakly virulent (PD-4), and avirulent (CB-1) strains of the Pierce's disease bacterium in inoculated grape petioles. Petioles were inoculated at time 0 with a drop of 10^7-10^8 cells per milliliter of bacterial suspension. ↓ = time of first appearance of leaf marginal necrosis symptoms with CB-9; symptoms were produced at 56 days with PD-4 and no symptoms were produced with CB-1.

Fig. 3. Movement of virulent (PD-1), weakly virulent (PD-4), and avirulent (PD-15) strains of the Pierce's disease bacterium up the stem of a Carignane grapevine after inoculation in a single internode. The third internode from the base of the plant was inoculated at time 0 with a drop of 10^7-10^8 cells per milliliter of bacterial suspension. The inoculated internode was designated as 0 and presence of bacteria in the various internodes was determined by culturing.
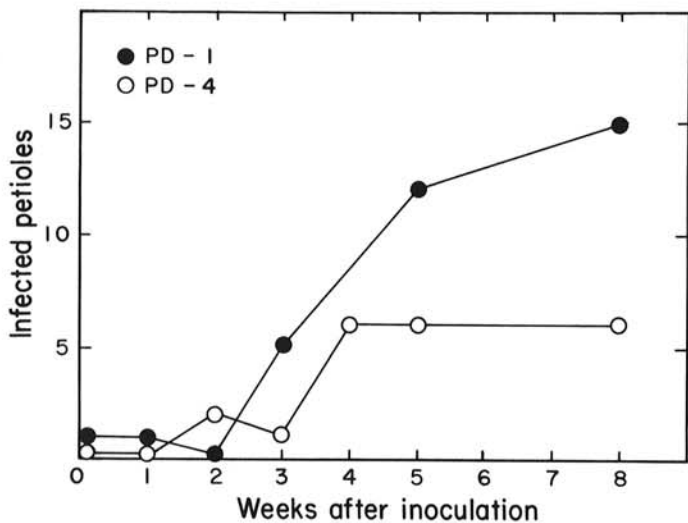
Fig. 4. Movement of virulent (PD-1) and weakly virulent (PD-4) strains of the Pierce's disease bacterium into petioles after inoculation of the third internode from the base of a Carignane grapevine. Inoculations were done at time 0 with a drop of 10^7-10^8 cells per milliliter of bacterial suspension and presence of bacteria in the various petioles above the inoculated internode was determined by culturing.

For cellulase assay, the bacteria were grown in liquid PD2 plus carboxymethylcellulose (8 g/L). Cellulase was not detected in any of the CFs. All tests were also negative for protease, aminopeptidase, and acid carboxypeptidase.

Peroxidase was detected in CFs from all of the PD strains and did not appear related to virulence. Peroxidase activity in these CFs varied from a 0.012 change in $A_{485\ nm}$ per minute with CB-1 to a 0.062 change with PD-12.

Cup-plate assays for phospholipase activity were positive for most of the CFs with lysolecithin as the substrate and negative with soybean lecithin as the substrate. Four virulent strains had an average assay rating of 2.75 for lysophospholipase (Table 1). Four weakly virulent strains had a rating of 1.75 and three avirulent strains had a rating of 0.67.

DISCUSSION

Virulence of PD strains in Carignane grapevine seemed related to both the rate of multiplication of bacteria in the tissue and the maximum bacterial populations attained. Even avirulent strains were able to infect the grapevine tissue and multiply. The virulence of PD strains was determined by their ability to multiply to threshold populations of approximately 10^6 cfu/cm in the inoculated grape petiole. Avirulent strains did not reach this threshold population before populations declined and, therefore, did not produce MN symptoms. Weakly virulent strains multiplied at a slower rate in the petiole than virulent strains, taking longer to reach the threshold population and to produce symptoms. Light microscopy of cross sections of inoculated petioles showed that virulent strains infested 10 times as many xylem vessels as did avirulent isolates (13). This difference could explain the failure of avirulent isolates to cause symptoms.

However, MN symptoms were highly correlated with PD bacterial concentrations in leaf veins and not with concentrations in petioles (11). The threshold population required in the petioles for MN production may be important primarily in providing bacteria for multiplication and accumulation in the leaf veins. Bacterial cells of avirulent strains rarely moved from the inoculated petiole into the leaf vein. Weakly virulent strains were slower to move into the leaf veins than were virulent strains (Fig. 2). Avirulent strains also failed to move out of the inoculated internodes of a grapevine, whereas virulent strains moved systemically through the grapevine and, after 3 mo, infested all but the newest growth (Fig. 3). These studies indicated that the lack of

virulence in PD strains resulted from the absence of the ability to multiply and move systemically through the susceptible grapevine.

Whether the primary factor in the loss of virulence is a failure of the bacteria to multiply sufficiently in the xylem vessels or a failure to move from vessel to vessel is not obvious. Both virulent and weakly virulent strains reached a population of 10^6 cells per centimeter of inoculated petiole prior to detection of bacteria in leaf veins, whereas avirulent isolates did not reach this level and were not detected in leaf veins. This would seem to indicate that reduced multiplication was the key to a loss of virulence. However, this reduced multiplication could actually be the result of a lack of movement from vessel to vessel. Perhaps, in the inoculated petioles, avirulent strains multiply in the vessels that they are pulled into during inoculation but are unable to move through pit membranes into adjacent vessels. This hypothesis is supported by the fact that initial multiplication rates of avirulent strains were nearly as fast as virulent strains, but avirulent populations in inoculated petioles peaked at 14 days and then declined.

Pit membranes in xylem vessels function as microfilters that allow the free flow of water in the transpiration stream but prevent large pathogens such as bacteria from passing between vessels. The rate of spread of pathogens within the xylem is limited by vessel length and the time required to dissolve an area of the pit membrane (19). The pit membrane is primary cell wall. I suspected that virulent strains must produce enzyme(s) that degrade the pit membrane, thus allowing vessel-to-vessel movement of the PD bacteria. However, I was unable to detect production of any pectolytic, proteolytic, or cellulolytic enzymes by any of the PD isolates. The mechanism of vessel-to-vessel movement, then, is still not known. Perhaps, PD bacteria can produce pectolytic, proteolytic, or cellulolytic enzymes in the host but not in our culture media. The PD bacteria produced peroxidase and lysophospholipase in culture and virulent strains produced more lysophospholipase than did avirulent ones. Phospholipases were assayed because of their possible effect on cell membranes, but the role, if any, of lysophospholipase in PD symptom development was not determined.

This study supports the hypothesis that leaf marginal necrosis symptoms result from blockage of xylem vessels producing water stress in the leaf (9,11) combined with phytotoxin action (14). Movement of PD bacteria into leaves and accumulation in leaf veins was consistently associated with virulence. Further studies are being done to evaluate the role of vessel blockage and phytotoxin in the PD syndrome.

LITERATURE CITED

1. Ayers, W. A., Papavizas, G. C., and Diem, A. F. 1966. Polyglacturonate trans-eliminase and polyglacturonase production by *Rhizoctonia solani*. Phytopathology 56:1006-1011.
2. Cooper, R. M., and Wood, R. K. S. 1975. Regulation of synthesis of cell wall degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. 5:135-156.
3. Davis, M. J., Purcell, A. H., and Thomson, S. V. 1978. Pierce's disease of grapevines: Isolation of the causal organism. Science 199:75-77.
4. Davis, M. J., Purcell, A. H., and Thomson, S. V. 1980. Isolation medium for the Pierce's disease bacterium. Phytopathology 70:425-429.
5. Dimond, A. E. 1970. Biophysics and biochemistry of the vascular wilt syndrome. Annu. Rev. Phytopathol. 8:301-322.
6. Doery, H. M., Magnusson, B. J., Gulasekharam, J., and Pearson, J. E. 1965. The properties of phospholipase enzymes in staphylococcal toxins. J. Gen. Microbiol. 40:283-296.
7. Goheen, A. C., Nyland, G., and Lowe, S. K. 1973. Association of a rickettsialike organism with Pierce's disease of grapevines and alfalfa dwarf and heat therapy of the disease in grapevines. Phytopathology 63:341-345.
8. Hildebrand, D. C. 1971. Pectate and pectin gels for differentiation of *Pseudomonas* sp. and other bacterial plant pathogens. Phytopathology 61:1430-1436.
9. Hopkins, D. L. 1977. Diseases caused by leafhopperborne, rickettsia-like bacteria. Annu. Rev. Phytopathol. 17:277-294.
10. Hopkins, D. L. 1980. Use of the pin-prick inoculation technique to demonstrate variability in virulence of the Pierce's disease bacterium. Pages 177-180 in: Proc. VIIth Int. Conf. Viruses of Grapevine (ICVG).

8-12 September 1980, Niagara Falls, Canada.

11. Hopkins, D. L. 1981. Seasonal concentration of the Pierce's disease bacterium in grapevine stems, petioles, and leaf veins. Phytopathology 71:415-418.
12. Hopkins, D. L., and Mollenhauer, H. H. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. Science 179:298-300.
13. Hopkins, D. L., and Thompson, C. M. 1981. Multiplication of virulent and avirulent Pierce's disease bacterial isolates in grapevine tissue. Pages 225-234 in: Proc. Vth Int. Conf. on Plant Pathological Bacteria. 16–22 August 1981, Cali, Colombia.
14. Lee, R. F., Raju, B. C., Nyland, G., and Goheen, A. C. 1982. Phytotoxin(s) produced in culture by the Pierce's disease bacterium. Phytopathology 72:886-888.
15. Mollenhauer, H. H., and Hopkins, D. L. 1976. Xylem morphology of Pierce's disease-infected grapevines with different levels of tolerance. Physiol. Plant Pathol. 9:95-100.
16. Nakadai, T., Nasuno, S., and Iguchi, N. 1972. Purification and properties of acid carboxypeptidase I from *Aspergillus oryzae*. Agric. Biol. Chem. 36:1343-1352.
17. Nakadai, T., Nasuno, S., and Iguchi, N. 1973. Purification and properties of leucine aminopeptidase I from *Aspergillus oryzae*. Agric. Biol. Chem. 37:757-765.
18. Wong, W. C., and Preece, T. F. 1978. *Erwinia salicis* in cricket bat willows: Peroxidase, polyphenoloxidase, β-glucosidase, pectinolytic, and cellulolytic enzyme activity in diseased wood. Physiol. Plant Pathol. 12:333-347.
19. Zimmerman, M. H., and McDonough, J. 1978. Dysfunction in the flow of food. Pages 117-140 in: Plant Disease, An Advanced Treatise. Vol. 3. J. G. Horsfall and E. B. Cowling, eds. Academic Press, New York.